Abstract: This study had the objective to provide a first evaluation of the potential antidiabetic and hypotensive activities of protein hydrolysates from spirulina, a protein-rich multicellular and filamentous cyanobacteria. The protein yield was enhanced by ultrasound assisted extraction and the preparation of pepsin (SP) and trypsin (ST) hydrolysates was optimised by a detailed kinetic study. A peptidomic analysis permitted to identify 55 and 76 species-specific peptides in the SP and ST hydrolysates, respectively. The prediction by the in silico tool BIOPEP that some of these peptides may be potential inhibitors of angiotensin converting enzyme (ACE) and peptidyl-peptidase IV (DPP-IV) was confirmed by experimental biochemical assays. Indeed, the SP and ST hydrolysates were effective ACE inhibitors, with IC50 values equal to 0.43 ± 0.01 mg mL⁻¹ and 1.36 ± 0.02 mg mL⁻¹, respectively, and DPP-IV inhibitors, with IC50 values equal to 3.4 mg mL⁻¹ and 3.0 mg mL⁻¹, respectively.
Dear Editor,

On behalf of all the authors, I have the pleasure to submit for publication the manuscript entitled: “CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF SPIRULINA PROTEIN HYDROLYSATES” by Gilda Aiello, Yuchen Li, Giovanna Boschin, Carlotta Bollati, Anna Arnoldi, Carmen Lammi.

Currently, microalgae are considered a fascinating topic, since they have demonstrated the potential to meet the population need for a more sustainable food supply, specifically with respect to protein demand. In this panorama, spirulina (Arthrospira platensis, synonym Spirulina platensis), with exceptionally high protein content (60-70% of its dry weight), is considered a promising protein source, not only for their nutritional value but also for the production of bioactive peptides with potential benefits for human health.

Based on these considerations, in the present work, we have investigated the anti-diabetic and anti-hypertensive activity of spirulina protein-derived hydrolysates. Briefly, total spirulina proteins have been extracted and hydrolyzed using pepsin and trypsin. Thus, the kinetics of peptides release from spirulina protein digested by the two enzymes have been investigated, in order to optimize the hydrolysis conditions. Afterwards, each hydrolysate was characterized by using an HPLC-ESI-MS/MS approach. In addition, the ability of both peptic and tryptic spirulina hydrolysates to modulate the activity of ACE and DPP-IV enzymes was assessed by performing in vitro experiments.

This submission represents an original work that has not been previously published and is not currently being considered by another journal. We declare no conflict of interest. Also, we confirm that all listed authors have contributed to the work and all authors have read, approved and agreed to submit the manuscript to Food Research International.

We hope that you can consider this manuscript suitable for publication.

Best wishes

Anna Arnoldi
Spirulina is a protein-rich multicellular and filamentous cyanobacteria
- Kinetics study of protein hydrolysis with pepsin and trypsin
- Peptidomic analysis of the pepsin and trypsin hydrolysates
- Evaluation of the ACE-inhibitory and DPP-IV inhibitory activities
CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF SPIRULINA PROTEIN HYDROLYSATES

Gilda Aiello, Yuchen Li, Giovanna Boschin, Carlotta Bollati, Anna Arnoldi,* Carmen Lammi

Department of Pharmaceutical Sciences, University of Milan, 20133 Milan, Italy

Abstract

This study had the objective to provide a first evaluation of the potential antidiabetic and hypotensive activities of protein hydrolysates from spirulina, a protein-rich multicellular and filamentous cyanobacteria. The protein yield was enhanced by ultrasound assisted extraction and the preparation of pepsin (SP) and trypsin (ST) hydrolysates was optimised by a detailed kinetic study. A peptidomic analysis permitted to identify 55 and 76 species-specific peptides in the SP and ST hydrolysates, respectively. The prediction by the in silico tool BIOPEP that some of these peptides may be potential inhibitors of angiotensin converting enzyme (ACE) and peptidyl-peptidase IV (DPP-IV) was confirmed by experimental biochemical assays. Indeed, the SP and ST hydrolysates were effective ACE inhibitors, with IC$_{50}$ values equal to 0.43 ± 0.01 mg mL$^{-1}$ and 1.36 ± 0.02 mg mL$^{-1}$, respectively, and DPP-IV inhibitors, with IC$_{50}$ values equal to 3.4 mg mL$^{-1}$ and 3.0 mg mL$^{-1}$, respectively.

Keywords: ACE inhibitor, bioactive peptides, DPP-IV inhibitor, kinetic of hydrolysis, LC-ESI-MS/MS, multifunctional peptides, peptidomics, spirulina proteins.

1. Introduction

Microalgae are composed of proteins rich of essential amino acids, fatty acids, antioxidant pigments, vitamins, and other bioactive compounds that express unique features for the production of pharmaceuticals, nutraceuticals, cosmetics, and biofuels (Spolaore, Joannis-Cassan, Duran, &
Isambert, 2006). In particular, there is a big interest for those species that contain great amounts of proteins providing functional properties (Soletto, Binaghi, Lodi, Carvalho, & Converti, 2005).

Among other species, spirulina (*Arthrospira platensis*, synonym *Spirulina platensis*) stands out due to its exceptionally high protein content (60-70% of its dry weight). Considering its health benefits, the World Health Organization declared it as “one of the greatest superfoods on the globe” and the United Nations supports an Intergovernmental Institution for the Use of Micro-Algae Spirulina Against Malnutrition (IIMSAM, Intergovernmental Observer to the United Nations Economic and Social Council Under ECOSOC Resolution 2003/212, dated 5th of March 2003) (Soni, Sudhakar, & Rana, 2017).

Currently, more and more researches are published on the nutraceutical properties of this microalga. After hydrolysis, the peptides released from spirulina protein exhibit a broad spectrum of biological effects, such as the anti-inflammatory (Romay, Gonzalez, Ledon, Remirez, & Rimbau, 2003), hypolipidemic (Torres-Duran, Ferreira-Hermosillo, & Juarez-Oropeza, 2007), hypoglycemic (Gargouri, Magne, & El Feki, 2016), anti-hypertensive (Torres-Duran, Ferreira-Hermosillo, & Juarez-Oropeza, 2007), antineoplastic (Mittal, Kumar, Banerjee, Rao, & Kumar, 1999), antiviral (Lee, Srisomporn, Hayashi, Tanaka, Sankawa, & Hayashi, 2001), antianemic (Simsek, Karadeniz, Kalkan, Keles, & Unal, 2009), and antioxidant activities (Karkos, Leong, Karkos, Sivaji, & Assimakopoulos, 2011).

The progress made in bioinformatics applied to food science and the knowledge of the amino acid sequences of food proteins permit to detect bioactive sequences and thus to monitor the potential release of bioactive peptides (Aiello, Lammi, Boschin, Zanoni, & Arnoldi, 2017; Ji, Han, Zhang, Hu, Fu, Qi, et al., 2018; Soni, Sudhakar, & Rana, 2017). Since the bioactivity of food-derived peptides depends on their physicochemical features, the information on the kinetic process by which these peptides are released is a crucial aspect to consider in order to produce hydrolysates with specific functionalities. In view of the scarce information available in literature on spirulina hydrolysis, the first objective of the work was to investigate the kinetics of peptides release from
spirulina protein digested by different enzymes, in order to optimize the experimental conditions.

The Michaelis-Menten kinetics model is a common approach for investigating the dynamic enzymolysis process, which is described by kinetic parameters (e.g., $K_m$, $K_{cat}/K_m$) that can contribute to understand the activity of the enzymes and even to optimize the hydrolysis conditions in further research (Jahanbani, Ghaffari, Vahdati, Salami, Khalesi, Sheibani, et al., 2018).

The second objective was to enlarge the knowledge on the biological activities of the obtained hydrolysates, focusing the attention on the antidiabetic and antihypertensive activity. The antidiabetic activity was explored testing their capability to inhibit peptidyl-peptidase IV (DPP-IV, EC 3.4.14.5), which is currently considered a new therapeutic target in type 2 diabetes, because it is able to degrade the incretins, including glucagon-like peptide-1 (GLP-1) and glucose inhibitory polypeptide (GIP), resulting in the loss of their ability to enhance insulin secretion (Carr, 2016; Nongonierma, Paolella, Mudgil, Maqsood, & FitzGerald, 2018). The hypotensive activity was instead investigated evaluating their ability to inhibit the activity of the angiotensin converting enzyme (ACE, EC 3.4.15.1), which is responsible for the conversion of the inactive decapeptide angiotensin I into the vasoconstrictor octapeptide angiotensin II, a major component of adverse blood pressure regulation (Fujita, Yokoyama, & Yoshikawa, 2000).

2. Materials and Methods

2.1. Reagents

All chemicals and reagents were of analytical grade. Acetonitrile (ACN), tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid (HCl), ammonium bicarbonate, pepsin from porcine gastric mucosa (P7012, lyophilized powder, ≥ 2,500 units/mg protein), trypsin from bovine pancreas (T1426, lyophilized powder, ≥ 10,000 units/mg protein), angiotensin converting enzyme (ACE) from porcine kidney, hippuryl-histidyl-leucine (HHL), formic acid, sodium chloride, zinc chloride, were from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) and β-mercaptoethanol were from Thermo Fisher Scientific (Life Technology, Milan Italy). Mini-Protean
apparatus, precision plus protein standards, Bradford reagent and Coomassie Blue G-250 were purchased from Bio-Rad (Hercules, CA, USA). LC-grade H$_2$O (18 MΩ cm) was prepared with a Milli-Q H$_2$O purification system (Millipore, Bedford, MA, USA).

2.2 Microalgae biomass

Spirulina dry powder was purchased from Qingdao Lang Yatai Company Limited (Qingdao, China). The manufacturer declares that they cultivate it in photoautotrophic conditions in outdoor runway pool and the dry powder is prepared by spray drying technology.

2.3 Ultrasound-assisted protein extraction from spirulina

Spirulina powder was defatted overnight with hexane (ratio 1:20 w/v) under magnetic stirring. After drying, the defatted powder was subjected to protein extraction. In details 0.5 g of defatted powder were suspended in 10 mL of NH$_4$Cl solution (0.05 M, pH 4.39). The mixture was treated with ultrasonic cell disruptor for 6 min, conducted for 5 s at 50 W, 23 kHz frequency pulses followed by 5 s of cool-down period in ice. Then the sonicated suspension was cleared via centrifugation at 7200 g at 4 °C for 30 min. The supernatant was collected and dialyzed against 0.01 M NH$_4$Cl using membranes and stored at -20 °C until analysis. The protein extraction protocol was evaluated by SDS-PAGE. The stacking gel was composed of a 4% polyacrylamide over a 12% resolving polyacrylamide gel. The cathodic and anodic compartments were filled with Tris–glycine buffer, pH 8.3, containing 0.1% m/v SDS. The electrophoresis was conducted at 100 V until the dye front reached the gel bottom. Staining was performed with colloidal Coomassie Blue and destaining with 7% (v/v) acetic acid in water.

2.4 Kinetics of the protein hydrolysis

The kinetics study of hydrolysis of spirulina proteins was performed to measure the initial reaction rate and the enzymolysis kinetics parameters ($K_m$ and $k_{cat}$) according to the following equation.
\[
V = \frac{K_{\text{cat}} \times [E]_0 \times [S]}{k_m + [S]}
\]

The reaction was initiated by adding 20 μg of each enzyme to 1 mL of protein solution at various concentrations (0.08, 0.2, 0.4, 0.8, 1.2, 1.6, 2, 3, 4, 5, 6 mg/mL). At time zero and after 60 min of incubation, aliquots of 5 μL were withdrawn and added to 200 μL of OPA. A Lineweaver–Burk plot was used to determine the kinetic parameters of the enzymolysis reaction. To determine the \(K_m\) and \(k_{\text{cat}}\), the equation was rearranged into a linear form. For experiments at a fixed enzyme concentration, the plot of \(1/V\) against \(1/S\) resulted in a straight line with slope \(K_m/k_{\text{cat}}\) and intercept \(1/k_{\text{cat}}\) allowing the calculation of \(K_m\) and \(k_{\text{cat}}\) values.

### 2.5 Spirulina protein hydrolysis for releasing bioactive peptides

The enzymatic hydrolysis of spirulina proteins was performed using trypsin and pepsin, dissolved in 1 mM HCl and 30 mM NaCl, respectively. For the trypsin digestion, the pH of the protein extracts was adjusted to pH 8 by adding 1 M NaOH and for the pepsin digestion it was set at pH 2-3 by adding 1 M HCl. The trypsin and pepsin solution were added to protein extracts at a 1:50 (w/w) E/S ratio. The reactions were mixed and incubated at 37 °C. For the kinetics study, 40 μL of each hydrolysis solution was pipetted out for blocking the reaction at 0, 5, 10, 20, 40, 60, 120, 180, 210 min incubation time points. After overnight incubation (16 h), all reactions were blocked: the samples digested by trypsin by heating at 95 °C for 5 min, whereas those digested with pepsin by adjusting the pH to 8. Each hydrolysate was passed through ultrafiltration (UF) membranes with a 3 kDa cut-off, using a Millipore UF system (Millipore, Bedford, MA, USA). All recovered peptides were lyophilized and store -80 °C until use. The degree of hydrolysis (DH) for each proteolytic system was detected by the o-phthaldialdehyde (OPA) assay. In the presence of β-mercaptoethanol, OPA reacts with the amino terminal of protein and peptides and the generated complex has a strong
absorbance at 340 nm. By measuring the absorbance of hydrolyzed and unhydrolyzed samples, the number of amino groups produced during the enzymatic reaction is measured. On this basis, the degree of hydrolysis (%) was calculated according to the equation:

\[ DH(\%) = \frac{\text{peptide content}}{\text{protein content}} \times 100 \]

2.6 Peptide sequencing by LC-ESI-MS/MS

Peptide profiles were obtained by LC-ESI-MS/MS analysis. Both peptic and tryptic hydrolysates were freeze-dried and reconstituted in 500 μL of a water solution containing 2% ACN and 0.1% formic acid. Four microliters of each hydrolysate were injected in a nanochromatographic system, HPLC-Chip (Agilent, Palo Alto, CA, USA). The analysis was conducted on a SL IT mass spectrometer (Agilent). Each sample was loaded onto a 40 nL enrichment column (Zorbax 300SB-C18, 5 μm pore size), and separated onto a 43 mm × 75 μm analytical column packed (Zorbax 300SB-C18, 5 μm pore size). Separation was carried out in gradient mode at a flowrate of 500 nL/min. The LC solvent A was 95% water, 5% ACN, 0.1% formic acid; solvent B was 5% water, 95% ACN, 0.1% formic acid. The nano pump gradient program was as follows: 5% solvent B (0 min), 50% solvent B (0–50 min), 95% solvent B (50–60 min), and back to 5% in 10 min.

Data acquisition occurred in positive ionization mode. Capillary voltage was −2000 V, with endplate offset −500 V. Full scan mass spectra were acquired in the mass range from m/z 300 to 2000 Da. LC-MS/MS analysis was performed in data-dependent acquisition AutoMS(n) mode. In order to increase the number of identified peptides, three technical replicates (LC-ESI-MS/MS runs) were run for each hydrolysate. The MS/MS data were analyzed by a Spectrum Mill Proteomics Workbench (Rev B.04.00, Agilent), consulting the A. platensis database (12530 entries) downloaded from the UniProtKB/Swiss-Prot - ExPASy. Pepsin and trypsin were selected as cutting enzyme. Two missed cleavages were allowed to each enzyme used; peptide mass tolerance was set to 1.0 Da and fragment mass tolerance to 0.8 Da. Autovalidation strategy both peptide and protein polishing mode was performed using FDR cut-off ≤ 1.2 %.
2.7 In vitro measurement of the ACE inhibitory activity

In order to evaluate the ACE-inhibitory activity, the hydrolysates were tested measuring the formation of HA from HHL, a mimic substrate for angiotensin I (Cushman & Cheung, 1971). The experimental details have been published elsewhere (Boschin, Scigliuolo, Resta, & Arnoldi, 2014; Boschin, Scigliuolo, Resta, & Arnoldi, 2014).

2.8 In vitro measurement of the DPP-IV inhibitory activity

The experiments were carried out in triplicate in a half volume 96 well solid plate (white) using conditions previously optimized (Lammi, Zanoni, Arnoldi, & Vistoli, 2016; Lammi et al, 2016). A total of 50.0 μL of each reaction was prepared in a microcentrifuge tube adding 30.0 μL of 1× assay buffer [20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, and 1 mM EDTA], 10.0 μL of SP or ST (at the final concentration of 1.0, 2.5, and 5.0 mg mL⁻¹), sitagliptin at 1.0 μM (positive control), or vehicle (C, H₂O) and 10.0 μL of purified human recombinant DPP-IV enzyme. Subsequently, reagents were transferred in each well of the plate and each reaction was started by adding 50.0 μL of substrate solution (5 mM H-Gly-Pro-AMC) and incubated at 37 °C for 30 min. Fluorescence signals were measured using the Synergy H1 fluorescent plate reader from Biotek (exc/em wavelengths 360/465 nm).

2.9 Statistical analysis of the biochemical assays

All measurements were performed in triplicate and results were expressed as the mean ± standard deviation, where p-values < 0.05 were considered to be significant. Statistical analyses were performed by one-way ANOVA (Graphpad Prism 7, GraphPad Software, La Jolla, CA, USA) followed by Dunnett’s test.

3. Results
3.1 Total protein extraction and assessment of \textit{in vitro} digestibility of spirulina proteins

Spirulina cells were lysed by ultrasound-assisted extraction, a viable method, recently used to improve the release of microalgae valuable components (Dey & Rathod, 2013). The efficiency of the extraction is illustrated in the Fig. 1 A, which displays the SDS-PAGE of the total protein extract (STPE lane) from spirulina powder. Two intense bands were detected in the STPE lane, corresponding to the $\beta$ (18.1 kDa) and $\alpha$ subunit (17.6 kDa) of C-phycocyanin (C-PC), which is the most abundant spirulina protein, accounting for 20% of the dry biomass. Most of the other proteins fell in the 25–100 kDa range, where the most intense bands were detected in the range between 55–35 kDa. The efficiency of the digestion process was evaluated comparing the protein and peptide profile of raw and enzymatically digested samples for both spirulina proteins digested by pepsin (SP) and trypsin (ST) as reported in Fig. 1B. As evident the susceptibility of total spirulina proteins to hydrolysis are quite different. C-PC resulted more prone to pepsin rather than trypsin digestion as revealed by comparing lane T$_0$ (SP) and lane T$_0$ (ST) (Fig. 1B). From the SDS-PAGE, it appears evident that practically all proteins, high molecular weight ones included, were completely hydrolysed during the enzymatic process. Fig. 1C indicates that the maximum rate of hydrolysis was achieved during the first 30 min for both enzymes and that more than one half of hydrolysis was accomplished after 3.5 h with DH equal to 23.4% and 24.4% for ST and SP, respectively. After overnight treatments, the DHs achieved were 37.8% and 49.4%, respectively, for SP and ST samples.

3.2 Kinetic study

The hydrolysis characteristics of pepsin and trypsin towards spirulina protein extract were investigated via Michaelis-Menten kinetics. For calculating the kinetic parameters $K_m$ and $k_{cat}$, the Lineweaver-Burk plots for both trypsin and pepsin hydrolysis were used as reported in Fig. 2. The calculated kinetics were $K_m = 4.33 \text{ mg/mL}$ and $k_{cat}$ of 0.72 min$^{-1}$ for pepsin and $K_m = 56.10 \text{ mg/mL}$
and $k_{\text{cat}} = 3.26 \text{ min}^{-1}$ for trypsin. Pepsin is thus a more efficient enzyme for spirulina protein hydrolysis.

### 3.3 Composition of spirulina protein hydrolysates

The whole genome of *A. platensis* has been sequenced and according to these data, the overall proteome has been predicted and annotated, to serve as the protein database for proteomic analysis. Using SwissProt UniProtKB *A. platensis* as reference database, a total of 48 and 55 protein sequences were identified with the HPLC-ESI-MS/MS, respectively for tryptic and peptic hydrolysate (*Table S1*). A total of 76 and 55 species-specific peptides were identified in the tryptic and peptic hydrolysates, respectively. Most of the trypsin-derived peptides belong to Allophycocyanin $\alpha$-$\beta$ chain, as well as to C-PC $\alpha$-$\beta$ chain, whereas the peptic hydrolysate did not contain any C-PC derived peptide. As already reported, the pH is a crucial parameter for the C-PC stability. C-PC keeps a good stability at pH 5-8, but it becomes unstable in acid environment ($\text{pH} \leq 4.5$) (Wu, Wang, Xiang, Li, & He, 2016). *Fig. 3A* reports the MW distribution in each profiled hydrolysate, whereas *Fig. 3B* indicates the distribution of peptides in each hydrolysate as a function of the peptide length and the hydrophobicity of each subgroup.

### 3.4 ACE inhibitory activity evaluated *in vitro*

The ACE inhibitory activity of the peptic and tryptic hydrolysates from spirulina are presented in *Fig. 4*. At the concentration of 1 mg mL$^{-1}$ the ACE inhibitions induced by SP and ST hydrolysates were $64.8 \pm 0.8\%$ and $46.3 \pm 0.7\%$, respectively (*Fig. 4*). The fact that the SP hydrolysate was more active than the ST hydrolysate was further highlighted by the IC$\text{50}$ values that are equal to $0.43 \pm 0.01$ mg mL$^{-1}$ and $1.36 \pm 0.02$ mg mL$^{-1}$, respectively.

### 3.5 DPP-IV inhibitory activity evaluated *in vitro*


In vitro experiments were carried out in order to evaluate the inhibitory activity of SP and ST peptides against human recombinant DPP-IV using the fluorescent substrate H-Gly-Pro-AMC. The enzymatic reaction was monitored by measuring the fluorescence signals due to the release of the free AMC group after the cleavage of the peptide H-Gly-Pro by DPP-IV and using sitagliptin (sita) at the final concentration of 1.0 µM, as positive control, which inhibited the enzyme activity by 88.9±1.9%. The activity of SP and ST were screened at the final concentration of 1.0, 2.5, and 5.0 mg mL\(^{-1}\). Bars in the Fig. 5 suggest that both SP and ST diminished the DPP-IV activity in vitro: SP dropped the enzyme activity by 15.2±0.9%, 42.0±1.7%, and 64.6±0.5% displaying an IC\(_{50}\) value equal to 3.4 mg mL\(^{-1}\) (Fig. 5A), while ST by 14.6±1.0%, 55.3±3.3% and 74.2±2.9% at 1.0, 2.5 and 5.0 mg mL\(^{-1}\), with an IC\(_{50}\) of 3.0 mg mL\(^{-1}\) (Fig. 5B), respectively.

4. Discussion

Spirulina stands out among other studied microalgae because it has been already certified as Generally Recognized as Safe (GRAS) by the Food and Drug Administration, thus enabling the application of peptides from this sources in food or drugs. Considering that the scientific studies on its antidiabetic and antihypertensive activities are limited, particularly in respect to peptides obtained by enzymatic hydrolysis, the aim of our work was to provide an evidence of the release of useful bioactive peptides. It is however important to premise that the limited information on the primary sequences of spirulina proteins limits the use of bioinformatic tools in predicting the pattern of bioactive peptides encrypted within their structure prior to experimental analysis (Ejike, Collins, Balasuriya, Swanson, Mason, & Udenigwe, 2017). Since the activity is strongly related to the structural features, the main goals of the work were: a) to optimize the hydrolysis conditions including choosing the best proteases according to the kinetics and DH parameters, b) to identify the sequences of tryptic and peptic peptides by LC-MS/MS based-proteomics, c) to assess their ACE and DPP-IV inhibitory activities.
The DH was used to monitor the enzymolysis process by pepsin and trypsin. The results indicated that a higher hydrolysis level was achieved with trypsin rather than with pepsin. This could be attributed to the fact that the different proteases have different restriction sites and can produce peptides with different molecular weight. Similar results have been reported previously (Koo, Bae, Lee, Lee, Hur, & Lee, 2014).

The spirulina protein digestibility was also described by a kinetics study. Km, the Michaelis-Menten constant, represents the substrate concentration at one half of the maximum hydrolysis velocity. The lower is the Km, the higher is the binding affinity between enzyme and substrate.

Another crucial parameter, the catalytic efficiency (kcat/Km), indicates the reaction products produced per unit of time. It also reveals how properly the substrate binds to the enzyme. In this case, pepsin was superior to trypsin either because it showed a better binding affinity or it had an improved catalytic efficiency, with a Km = 4.33 mg/mL and kcat of 0.72 min⁻¹, whereas the trypsin parameters were Km = 56.10 mg/mL and kcat = 3.26 min⁻¹. On the opposite, the tryptic hydrolysate exhibited a higher DH. Actually, a kinetic study tends to be focused on the hydrolysis characteristics of the enzyme towards a certain substrate. The kinetic parameters calculated from the kinetic model are aimed to illustrate the specific enzyme efficiency rather than to focus on the final produced peptides, whose quality is measured by the DH. The latter depends on the enzyme and initial substrate concentration and the time (Hu, Ci, Wang, Zhang, Zhang, Thakur, et al., 2018; Zhou, Yu, Qin, Ma, Yagoub, & Hu, 2016). The different restriction sites of enzymes and some complex factors, such as enzyme deactivation, can also affect the final DH (Ghadge, Patwardhan, Sawant, & Joshi, 2005). For these reasons, there is not a strict correlation between the DH and the kinetic parameters through which peptides are produced.

Overall, this study provided for the first time, the kinetics information on the enzymatic activity of pepsin and trypsin towards spirulina proteins. Both the kinetics study and the DH detection are
useful to describe and to understand the spirulina protein hydrolysis process as well as to optimize the hydrolysis conditions in further research.

4.2 Peptidomic analysis

The characterization of the whole peptide profiles showed the different identified sequences, deriving from the most abundant proteins. The most frequently detected peptides in ST derived from C-PC, both $\alpha$ and $\beta$ subunit covered about 37% and 35%, respectively, of protein sequences. On the contrary, the identified peptide in the peptic hydrolysates did not belonged to C-PC. This findings could be attributed to two phenomena: the C-PC precipitation in acidic conditions (Minic, Stanic-Vucinic, Mihailovic, Krstic, Nikolic, & Velickovic, 2016) and/or the very fast digestion by pepsin (Minic, Stanic-Vucinic, Mihailovic, Krstic, Nikolic, & Velickovic, 2016), which led to very small peptides undetectable by our MS method. Here, it is important to underline that the MS/MS spectra elaborated by SpectrumMill search engine allowed the identification of peptide sequences with a number of amino acids residues higher than 4, whereas smaller peptides, such as di-tri and tetrapeptides, cannot be uniquely assigned to a specific protein. The molecular weights of the released peptides, both peptic and tryptic, fell between 1.5 and 1 kDa as reported in Fig. 3A. The spectral counting quantification, which relies on the number of peptides identified from a given protein, revealed that the most abundant proteins detected in the tryptic hydrolysate belong to Allophycocyanin and C-phycocyanin, followed by 60 kDa chaperonin, Elongation Factor Tu, and Fructose-bisphosphate aldolase. The most abundant proteins detected in the peptic hydrolysates were instead not yet characterized species, whose biological functions are still unknown. Apparently, the composition and the distribution of peptides among the expressed spirulina proteome, according to DH, seems to direct the choice towards trypsin as the best proteolytic enzyme, since it performs better in the hydrolysate production.

4.3 Bioactivity investigations towards diabetic and cardiovascular diseases
In recent years, bioactive food-derived peptides have received increasing attention for their health benefits particularly in the area of cardiovascular diseases (de Campos Zani, Wu, & Chan, 2018). In order to screen the effects of spirulina peptides on ACE and DPP-IV activity, in vitro assays were employed.

Our findings suggest that both SP and ST peptide mixtures are able to drop the ACE activity in vitro with IC₅₀ values equal to 0.43 ± 0.01 mg mL⁻¹ and 1.36 ± 0.02 mg mL⁻¹, respectively (Fig. 4). The value of the ST peptides is in agreement with previous ones where the hydrolysis of spirulina proteins had been conducted with other enzymes. In particular, the hydrolysate obtained after digestion with alcalase inhibited the ACE activity with an IC₅₀ value equal to 0.23 mg mL⁻¹ (Lu, Ren, Xue, Sawano, Miyakawa, & Tanokura, 2010), whereas spirulina peptides, generated after the hydrolysis with Protamex and SM98011, inhibited the ACE activity with an IC₅₀ value smaller than 0.5 mg ml⁻¹ (He, Chen, Wu, Sun, Zhang, & Zhou, 2007). The activity of the ST peptides was instead comparable to that of the peptides mixtures produced by alcalase and flavourzyme with IC₅₀ values in the range of 1.0 - 2.0 mg mL⁻¹ (He, Chen, Wu, Sun, Zhang, & Zhou, 2007). Literature reports also some in vivo studies. Recent evidences have demonstrated that the oral administration of a peptidic fraction (200 mg kg⁻¹) derived by peptic hydrolysis of spirulina in spontaneously hypertensive rats (SHRs) caused a significant reduction to 39.5 mmHg at 2 h, with a continuous antihypertensive effect for 4 h (Suetsuna & Chen, 2001). Specifically, peptides AQL, IAPG, and VAF detected in this study showed IC₅₀ values equal to 34.7 μM, 11.4 μM and 35.8 μM, respectively.

The higher ACE inhibitory activity of the peptic peptides in respect to the tryptic peptides suggests that their different composition and physical-chemical properties influence their bioactivity. For this reason, the distribution of peptides in each hydrolysate as a function of the peptide length was determined and the hydrophobicity of each subgroup was calculated. More in detail, the SP hydrolysate contained 27.3% peptides with a 7-10 amino acid residues length and a hydrophobicity of 14.4 kcal mol⁻¹, 38.2% peptides with a length of 11-15 amino acid residues and a hydrophobicity...
of 16.7 kcal mol\(^{-1}\), and 34.5% peptides with a length of 16-23 amino acid and a hydrophobicity of 20.7 kcal mol\(^{-1}\) (Fig. 3B). On the contrary, the ST hydrolysate contained 18.4% peptides with a length ranging from 7 to 10 amino acid residues and a hydrophobicity of +14.5 kcal mol\(^{-1}\), 36.8% peptides with 11-15 amino acid residues length and a hydrophobicity of 16.4 kcal mol\(^{-1}\), and 44.8% of peptides with a length of 16-27 amino acid and a hydrophobicity of 20.9 kcal mol\(^{-1}\) (Fig. 3B).

Since a high hydrophobicity value corresponds to a great number of hydrophobic amino acids in the peptide sequence, it is possible to affirm that both SP and ST mixtures contained a large amount of hydrophobic peptides even though the shortest peptides were more abundant in the SP hydrolysate than in the ST one. This important difference may possibly explain why the SP mixture was a more efficient ACE-inhibitor.

Furthermore, it is well established that, in order to be a good ACE inhibitor, a peptide with 2-10 amino acid residues length should contain numerous hydrophobic amino acid residues as well as a relative abundance of specific amino acids, such as Pro, His, Glu and Asp (Daskaya-Dikmen, Yucetepe, Karbancioglu-Guler, Daskaya, & Ozcelik, 2017). Moreover, the ability of a peptide to binds the ACE is highly influenced by the C-terminal region (Castellano, Aristoy, Sentandreu, Vignolo, & Toldrá, 2013; Fujita, Yokoyama, & Yoshikawa, 2000). In facts, a common feature of good ACE-inhibitory peptides is the presence of residues with aromatic (Tyr, Trp, and Phe), positive charge (His, Arg, Lys), or branched (Leu, Ile, and Val) side chains located in the C-terminal region (Mine & Kovacs-Nolan, 2006). Taking all these observations in account and using the BIOPEP database as a tool to predict potential bioactivity, four peptides in the SP hydrolysate (IQKPGSVIR, IKDKNRTPFN, FQAAGHSVAL, and FGSATQVAN) and five peptides in the ST one (YLSPGELDR, DLDYYLR, AYFATGELR, ALAVSELYR, and TDLPHVIHR) seem to satisfy these features and may thus be the most active ACE inhibitory species in these hydrolysates (Table 1).

Interestingly, the SP and ST hydrolysates display a multifunctional behavior, since they also decrease the DPP-IV activity \textit{in vitro}. In particular, tested at 1.0, 2.5, and 5.0 mg mL\(^{-1}\)
concentrations, the SP hydrolysate reduced the enzyme activity by 15.2 ± 0.9%, 42.0 ± 1.7%, and 64.6 ± 0.5% (Fig. 5A), whereas the ST one by 14.6 ± 1.0%, 55.3 ± 3.3%, and 74.2 ± 2.9%, respectively (Fig. 5B). Thus, our data confirm the results of a previous study that have predicted the potential DPP-IV inhibitory activities of the tryptic and peptic peptides generated from spirulina proteome only using BIOPEP (Ji, et al., 2018). Moreover, the results of the present study are in line with the bioactivity of other food protein hydrolysates (Power, Nongonierma, Jakeman, & FitzGerald, 2014). In particular, an Atlantic salmon skin gelatin hydrolysate, obtained using Flavorzyme®, diminished the DPP-IV activity by 45.0% at 5.0 mg mL⁻¹ (Li-Chan, Hunag, Jao, Ho, & Hsu, 2012), whereas, a tryptic hydrolysate of amaranth proteins had an IC₅₀ values between 1.2 and 2.0 mg mL⁻¹ (Velarde-Salcedo, Barrera-Pacheco, Lara-Gonzalez, Montero-Moran, Diaz-Gois, Gonzalez de Mejia, et al., 2013). Moreover, a Japanese rice bran peptide mixture obtained using Umanizyme G® reduced the DPP-IV activity with an IC₅₀ equal to 2.3 mg mL⁻¹ (Hatanaka, Inoue, Arima, Kumagai, Usuki, Kawakami, et al., 2012).

Even though the bioactivity of ST and SP hydrolysates is in line with the bioactivity of other food-derived hydrolysates, from a methodological point of view it is important to underline that our study was assessed using the human recombinant DPP-IV enzyme, whereas the other studies have been carried out using the porcine DPP-IV enzyme. Although the sequence is highly conserved among mammalian species, human and porcine DPP-IV differ in their susceptibility to inhibition by food-derived peptides (Lacroix & Li-Chan, 2015). Since the inhibition is stronger on the porcine DPP-IV enzyme than on the human one, the employment of the former to assess the inhibitory effect may lead to an overestimation of the actual potency or effectiveness of a sample (Lacroix & Li-Chan, 2015).

The structure-activity relationship (SAR) analysis of food-derived peptides with DPP-IV inhibitory activity suggests that hydrophobic peptides with 2-8 amino acid residues length and containing Pro residues (at the first, second, third, or fourth N-terminal position), flanked by Leu, Val, Phe, Ala, and Gly, display a good DPP-IV inhibitory property (Lammi, Zanoni, Arnoldi, & Vistoli, 2016).
In this context, three peptides from soybean (IAVPTGVA, IAVPGEVA, and LPYP) and one from lupin (LTFPGSAED) have been identified that have the ability to drop the DPP-IV activity in vitro on human purified DPP-IV enzyme, in situ on human intestinal Caco-2 cells, and ex vivo on human serum (Aiello, Ferruzza, Ranaldi, Sambuy, Arnoldi, Vistoli, et al., 2018; Lammi, Bollati, Ferruzza, Ranaldi, Sambuy, & Arnoldi, 2018; Lammi, Zanoni, Arnoldi, & Vistoli, 2016). In light with these observations, among the peptides containing less than 10 amino acid residues detected in the SP and ST hydrolysates, peptides IQKPGSVIR, LEQPLGVNE, MPTSQDQK, LPDATETKM, IEQVKPGVE, TPSDFEYNVR, YLSPGELDR, and TDLPHVIHR satisfy these structural features. As shown in the Table 3, all these peptides exhibit hydrophobicities ranging from +12.33 to +17.98 kcal mol⁻¹ and are proposed as potential DPP-IV inhibitors by BIOPEP informatics tool, which suggests that they might display multifunctional properties being also potential ACE inhibitors (Table 1). In this context, other food-derived peptides with this multifunctional behavior are reported in literature. In particular, LPYPY from milk is a DPP-IV inhibitor with an IC₅₀ equal to 90.8 µM (Nongonierma, Mooney, Shields, & FitzGerald, 2014) and an ACE inhibitory activity with an IC₅₀ equal to 28.9 µM (Gomez-Ruiz, Ramos, & Recio, 2007). Based on these evidences, further efforts will be pursued in order to confirm these predictions and also to assess whether these peptides may be absorbed at intestinal levels.

5. Conclusion

By the combination of high-throughput proteome analysis and in vitro bioactivity assays, this work provides information which support the development of functional foods or dietary supplements from spirulina. These results may have impact in different fields of applications, starting from the selection of the best hydrolytic conditions and followed by the MS-peptide sequencing useful for elucidating structure-activity relationships based on Quantitative Structure-Activity Relationship (QSAR) models. In addition, this study demonstrates for the first time the potential antidiabetic properties of spirulina hydrolysates. The production of spirulina-derived, bioactive-rich functional
foods with antidiabetics and antihypertensive properties may contribute to the ongoing efforts to reduce the global cardiovascular disease (CVD) burden.

**AUTHOR INFORMATION**

**Corresponding Author**

Anna Arnoldi, Department of Pharmaceutical Sciences, University of Milan, via Mangiagalli 25, 20133 Milan, Italy. E-Mail: anna.arnoldi@unimi.it, tel.: +390250319342.

**Author contributions**

Experiment ideation and design, G.A. and Y.L.; Biological experiments, C.L. C.B. and Y.L. G.B.; Data analysis, G.A. C.L. and Y.L.; Discussion of the results, G.A. C.L. and Y.L., Manuscript writing, G.A, Y.L. C.L. and A.A.

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**ABBREVIATIONS USED**

ACE, angiotensin converting enzyme; DPP-IV, peptidyl-peptidase IV; IIMSAM, Intergovernmental Institution for the Use of Micro-Algae Spirulina Against Malnutrition; GLP-1, glucagon-like peptide-1; GIP, glucose inhibitory polypeptide; ACN, Acetonitrile; Tris, tris(hydroxymethyl)aminomethane; HHL, hippuryl-histidyl-leucine; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UF, ultrafiltration; OPA, o-phthaldialdehyde; DH, degree of hydrolysis; LC-ESI-MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry; HA, hippuric acid; IB, inhibitor blank; RB,
reaction blank; IC$_{50}$, inhibitory concentration 50%; C-PC, C-phycocyanin; SP, Spirulina proteins digested by pepsin; ST, Spirulina protein digested by trypsin; MW, molecular weight; GRAS, Generally Recognized as Safe; SHRs, spontaneously hypertensive rats. QSAR, quantitative structure-activity relationship; CVD, Cardiovascular.
References


Figure 1. Protein profile, degree of hydrolysis (DH) trend, and digestion efficiency of spirulina protein. A) SDS-PAGE pattern of spirulina total protein extract (STPE). Lanes number 1 and 2 are two independent extractions. B) SDS-PAGE analysis of hydrolysates sampled at different hydrolysis time points. SP: spirulina proteins digested by pepsin, ST: spirulina protein digested by trypsin. C) DH at different time points within the first 3.5 h of in vitro digestion.

Figure 2. Lineweaver-Burk plots of spirulina protein hydrolysis by pepsin and trypsin.

Figure 3. A) Molecular weight distribution of peptides identified in hydrolysate fraction less than 3 kDa in SP and ST groups. B) Length and a hydrophobicity distribution among the hydrolysate ST and SP.

Figure 4. ACE inhibition curves at different concentrations of ST and SP peptide.

Figure 5. DPP-IV inhibitory activities of SP (A) and ST (B) peptides. Bars represent the average ± SEM of 3 independent experiments in duplicate. ***p<0.001 **p<0.01 versus untreated sample (C). Sitagliptin at 1.0 μM was used as positive control.
Figure 3
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Figure 4

The graph shows the % ACE inhibition plotted against peptide concentration (µg/mL). Two lines are depicted: one for ST (red squares) and one for SP (blue triangles). The graph indicates a dose-dependent inhibition of ACE activity, with percentage inhibition increasing with higher peptide concentrations.
Table 1. Net charge, hydrophobicity, and potential bioactivity of peptic (A) and tryptic (B) peptides from Spirulina protein hydrolysis.

<table>
<thead>
<tr>
<th>Protein name a)</th>
<th>Peptide sequence b)</th>
<th>Net Charge c) (#)</th>
<th>Hydrophobicity (kcal/mol) c)</th>
<th>DPP-IV Inhibitory Peptides d)</th>
<th>ACE Inhibitory Peptides d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncharacterized protein</td>
<td>(E)IQKPGSVIR(I)</td>
<td>2</td>
<td>12.33</td>
<td>KP, IQ, IR, PG, SV, VI</td>
<td>IR, GS, PG, QK, KP</td>
</tr>
<tr>
<td>o-succinylbenzoate synthase</td>
<td>(F)LEQPLGVNE(L)</td>
<td>-2</td>
<td>15.11</td>
<td>QP, PL, GV, NE, VN</td>
<td>PLG, PL, GV, LG, LGV</td>
</tr>
<tr>
<td>Two-component sensor histidine kinase</td>
<td>(-)MPTSQDQK(I)</td>
<td>0</td>
<td>16.06</td>
<td>MP, DQ, PT, QT, DS</td>
<td>QK, PT</td>
</tr>
<tr>
<td>WD-40 repeat protein</td>
<td>(L)IKDKNRTPFN(I)</td>
<td>2</td>
<td>18.21</td>
<td>------------</td>
<td>TP</td>
</tr>
<tr>
<td>Uncharacterized protein</td>
<td>(Y)FQAAGHSVAL(Y)</td>
<td>0</td>
<td>10.69</td>
<td>------------</td>
<td>AA, AG, GH, FQ</td>
</tr>
<tr>
<td>Aminopeptidase P</td>
<td>(C)IEQVKPGVE(Y)</td>
<td>-1</td>
<td>17.98</td>
<td>KP, GV, PG, QV, VE, VK</td>
<td>VK, GV, PG, VKP, VE, IE, KP</td>
</tr>
<tr>
<td>Uncharacterized protein</td>
<td>(S)LPDATETKM(I)</td>
<td>-1</td>
<td>17.09</td>
<td>LP, AT, ET, TE, TK</td>
<td>DA, TE</td>
</tr>
<tr>
<td>Uncharacterized protein</td>
<td>(D)FGSATQVAN(F)</td>
<td>0</td>
<td>10.21</td>
<td>------------</td>
<td>FG, GS, TQ</td>
</tr>
</tbody>
</table>
### Table 1: Predicted ACE and DPP-IV Inhibitory Peptides

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Peptide Sequence</th>
<th>Net Charge ($#$)</th>
<th>Hydrophobicity (kcal/mol)</th>
<th>DPP-IV Inhibitory Peptides</th>
<th>ACE Inhibitory Peptides $^{d}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allophycocyanin beta chain</td>
<td>(K)AYFATGELR(V)</td>
<td>0</td>
<td>12.07</td>
<td></td>
<td>YL, DY, LR, LDY</td>
</tr>
<tr>
<td></td>
<td>(R)DLDYYLR(Y)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase</td>
<td>(K)TPSDFEYNVR(V)</td>
<td>-1</td>
<td>15.8</td>
<td></td>
<td>VR, Y, TP, DE, YN</td>
</tr>
<tr>
<td>Phycobilisome rod Linker polypeptide CpcH</td>
<td>(R)ALAVSELYR(K)</td>
<td>0</td>
<td>11.13</td>
<td></td>
<td>LY, LA, AV</td>
</tr>
<tr>
<td>Mannose-1-phosphate guanylyltransferase/phosphomannomutase</td>
<td>(R)TDLPHVIHR(R)</td>
<td>0</td>
<td>15.57</td>
<td>LP, HR, HV, IH, PH, DT, VI</td>
<td>DLP, PH</td>
</tr>
</tbody>
</table>

$^{a}$ Protein name, $^{b}$ Peptide sequence, $^{c}$ Net charge (sum of positively (basic) and negatively (acidic) charged residues at neutral pH) and hydrophobicity (Wimley-White scale) of peptide sequences were predicted using PepDraw tool at http://www.tulane.edu/~biochem/WW/PepDraw/. $^{d}$ Data accessed from BIOPEP database, available at http://www.uwm.edu.pl/biochemia/index.php/pl/biopep on July 2018.
Click here to download Supplementary material for online publication only: Table S1.docx
S. platensis

Chemical analysis

Biological investigation

ACE and DPP-IV inhibitory activities

MS/MS profiles and DH trends

*Graphical Abstract